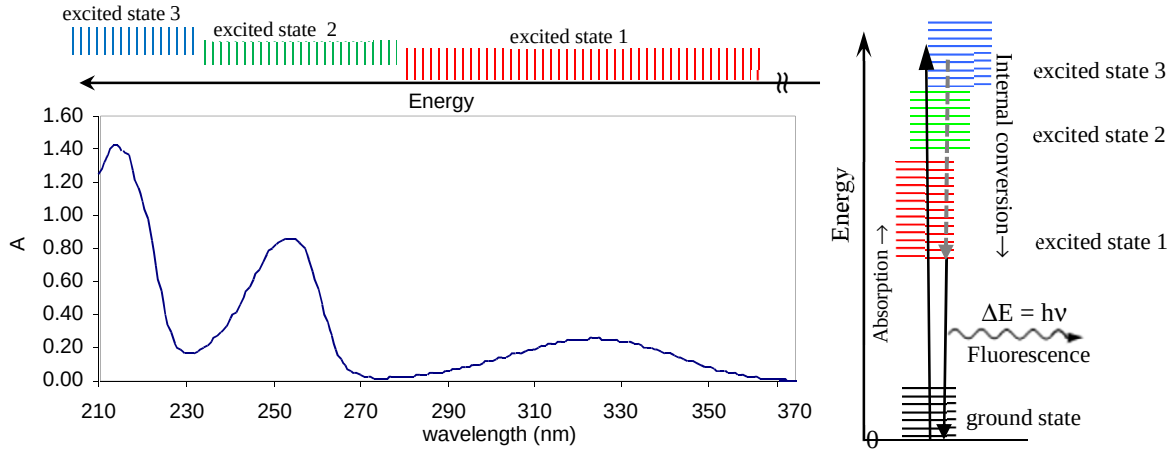
Fluorescence Quenching of Fluorescein [1]

Purpose

This experiment utilizes fluorescence intensity measurements to determine the quenching constant for the fluorescence quenching of fluorescein by iodide ion in basic solution. The source of fluorescein is a commercial text highlighter.

Introduction

The absorption spectrum for an example molecule is shown in Figure 1. The short wavelength side of the spectrum corresponds to the bluer side of the spectrum and the long wavelength side is the redder end of the spectrum. For this example molecule the absorption bands are all in the ultra-violet region. Three absorption transitions, or bands, are observed. These three absorption transitions correspond to three excited states, Figure 1b. The energy level diagram is essentially the absorption spectrum converted to an energy scale and then tilted by 90 degrees, with the molecular energy levels shown as bands (series of parallel horizontal lines). What happens to the excess energy after a molecule absorbs light? Fluorescence results from the first, lowest energy excited state. The first excited state is also deactivated by nonradiative processes, which decrease the fluorescence intensity. Fluorescence and nonradiative processes are in competition. Much of the nonradiative deactivation is by collisions of the excited molecule with solvent molecules. However, some substances are particularly efficient at deactivating, or quenching, excited states. These substances often contain heavy atoms or are magnetic compounds. Oxygen is a particularly good quencher, therefore it is often necessary to remove the oxygen from solution before measuring fluorescence spectra. Fluorescent probes are used in biochemistry to study the binding sites in large macromolecules through the difference of the quenching rates of the bound verses free probe.



(a) (b)

Figure 1: (a) The absorbance spectrum shows three absorption bands corresponding to three molecular excited states. (b) The three excited states are shown in the corresponding energy level diagram. Fluorescence results from the lowest energy excited state even if the excitation is to higher energy excited states. (There is no horizontal axis for an energy level diagram.)

Theory

The Stern-Volmer Mechanism: When ultraviolet light shines on a solution of fluorescein, molecules are promoted to an excited state, Figure 1b. If the excited state is not the lowest excited state, the molecule rapidly drops into the lowest excited state through **internal conversion**. In internal conversion, the excess energy is lost as heat. The molecule in the first excited state is denoted by A* and can do one of several things. The excited state molecule can fluoresce from the lowest energy excited state and be converted back to the ground state. The rate is proportional to a constant, k_f.

$$A^* \xrightarrow{k_f} A + h\nu$$

A second possibility is for the excited molecule to lose energy in the form of heat rather than light. This **nonradiative** process is internal conversion to the ground state. The rate is proportional to a constant, k_{nr}:

$$A^* \xrightarrow{k_{nr}} A + \text{heat}$$

The intensity, I^o, for fluorescence in pure solvent is proportional to the rate of fluorescence divided by the rate of fluorescence and nonradiative decay:

$$I^o = \frac{\text{fluorescence}}{\text{fluorescence + nonradiative}} = \frac{k_f}{k_f + k_{nr}} \qquad (1)$$

If I^- is present in the solution, a third mechanism for the excited molecules to become depleted is available, quenching. Q**uenching** by I^- depends on the concentration of I^-. More I^- causes a faster loss of the excited state without emission of light. The rate is proportional to a constant multiplied by the I^- concentration, $k_q[I^-]$:

$$A^* + I^- \xrightarrow{k_q[I^-]} A + I^- + \text{heat}$$

Quenching results in the ground state molecule and heat. The net effect of the presence of I^- is a reduction in the intensity of fluorescence to give:[2]

$$I = \frac{\text{fluorescence}}{\text{fluorescence + nonradiative + quenching}} = \frac{k_f}{k_f + k_{nr} + k_q[I^-]} \qquad (2)$$

Dividing I^o by I gives the relative intensity, which yields the Stern-Volmer expression:[3]

$$I^o/I = 1 + \frac{k_q}{k_f + k_{nr}}[I^-] \qquad (3)$$

The constants taken together are called the quenching constant, $K_q = k_q/(k_f + k_{nr})$ giving:

$$I^o/I = 1 + K_Q[I^-] \qquad (4)$$

A plot of I^o/I versus $[I^-]$ yields a straight line with a slope K_Q. The quenching constant is a comparison of the rate of quenching and the rate of formation of the first excited state. The intercept is expected to be one to within experimental error. This process is called the Stern-Volmer mechanism.

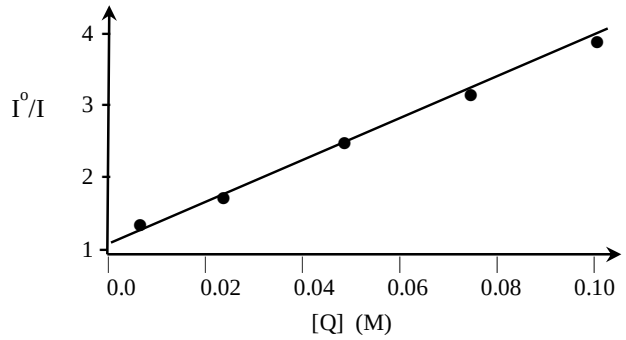


Figure 1: Stern-Volmer plot for fluorescence quenching.

Static and Dynamic Quenching: Two types of quenching mechanisms are commonly found.[4] **Dynamic** quenching is described by the Stern-Volmer mechanism and results by collisions of the excited state molecule with the quencher. **Static** quenching involves the interaction of the ground state molecule and the quencher. Unfortunately, linear plots can result from both static and dynamic quenching. Absorbance spectroscopy is a probe primarily of the ground state properties of the molecule. Changes in the absorbance spectrum upon addition of quencher provides support for the static mechanism. Quenching without changes in the absorbance spectrum provides support for interaction of the quencher with the excited state through the Stern-Volmer mechanism.

Equipment: Supplies

Vernier SpectroVis Plus
3xPlastic cuvettes with frosted sides for absorbance measurement
6xPlastic fluorescence cuvettes (four clear sides)
5 x 10 ml volumetric flasks
1 x 25 ml volumetric flask (for KI stock solution)
Automatic pipettor: 1000 µl
Fluorescent yellow text highlighter: fluorescein
150 mL-beaker
KI
0.1 M pH 7.4 buffer (11.03 g Na_2HPO_4 and 3.05 g KH_2PO_4 in 1 L water)
Ethanol, 95%

Procedure

The outline of the procedure is:

1. Prepare stock solutions of fluorescein and 1.0 M KI in water. The fluorescence of fluorescein is dependent on OH^- concentration so the fluorescein stock solution is made up in a buffer solution that maintains constant [OH^-] (that is constant pH).
2. Determine the absorption spectrum of fluorescein in water and I^- solution to look for ground state interaction between the fluorescing molecule and the quencher.
3. Determine the wavelength range of the light source at 405 nm that is used for fluorescence excitation.
4. Determine the fluorescence intensity of the dye alone and in the presence of increasing concentration of I^- as quencher.

1. *Preparation of the Fluorescein and Iodide Stock Solutions*: Use a yellow text highlighter to cover both sides of a ~2.0 cm x 10 cm strip of paper. Place the yellow paper strip in a 150-mL beaker with roughly 100 mL of pH 7.4 buffer. Stir using a stir bar on a magnetic stirrer for a few minutes until the dye is mostly desorbed from the paper. The absorbance of the final solutions for fluorescence intensity determination must not exceed 0.2 so that the fluorescence intensity is uniform throughout the solution. Accordingly, measure the absorbance of your stock solution of fluorescein (using a frosted sided cuvette). The absorbance at the wave length of maximum absorption for the transition near 450 nm should be in the 0.4-0.7 range. If the stock solution is too concentrated, dilute with buffer. This stock solution is diluted by a factor of ten in making up the final solutions, so the absorbance will be in the target range upon completion.

Prepare a 1.0 M KI solution in reagent grade water in a 25-mL volumetric flask using the following steps:

1. Weigh out the required amount of solid KI in a 20-mL beaker. The amount of KI needed is calculated to give a final concentration in the range of 0.9-1.1 M. Record the mass of KI that you use to at least two-significant figures past the decimal point.
2. Add about 10-12 mL of water, stir with a stirring rod or small spatula, remembering to keep the stirring rod <u>in the beaker at all times</u> to avoid loss of KI. (An ultrasonic bath may be used to hasten dissolution).
3. Use a small plastic funnel to transfer the KI solution into the volumetric flask. Taking care not to go over the calibration mark on the volumetric flask, rinse the beaker with several small additions of water to quantitatively transfer all the KI into the volumetric flask.
4. Dilute to the mark using a plastic dropper. The <u>bottom</u> of the solution meniscus should be at the calibration mark.

2. *Absorbance Measurements*: Using a auto-pipettor, add 1 mL of stock dye solution and 1 mL of water into a plastic cuvette (using a frosted sided cuvette). In a second cuvette add 1 mL of stock dye solution and 1 mL of 1.00 M KI. Cover each cuvette in turn with a piece of plastic wrap or parafilm and shake to mix the solutions. Determine the absorbance spectrum of the two solutions using the attached instructions. Are any significant changes in the absorption spectrum observed upon addition of I^- ion? Small changes in intensity might result from the relatively crude manner of solution preparation.

3. *Determination of the Excitation Profile at 405 nm*: Switch the SpectroVis Plus to fluorescence mode by pulling down the Experiment menu, sliding right on “Change units” and “Spectrometer 1,” and choosing “Fluorescence 405 nm.” We first want to determine the range of wavelengths for the excitation. The location of the purple LED used for this purpose is indicated on the top of the instrument, on the side of the cuvette holder. Reflecting the excitation light into the spectrometer would overwhelm the detector. Instead, dilute one small drop of coffee creamer in a small beaker of water. This suspension should be only slightly turbid. Transfer some of the creamer suspension into a frosted sided cuvette. Determine the spectrum using the attached instructions. Compare the absorption spectrum of the dye and the profile of the excitation at 405 nm. Is there significant overlap between the molecule absorption and the fluorescence excitation? Pull down the Data menu and choose Delete All.

4. *Fluorescence Measurements*: Five different dilutions of the KI solution with constant dye concentration are to be prepared along with one solution with dye only to determine I^o. Pipet 1.00 ml of fluorescein stock solution into each of six 10-mL volumetric flasks. Pipet 2.00 mL of ethanol into each flask. Set aside the first solution. Pipet 1, 2, 3, and 4 mL of KI stock solution

into the remaining 10-ml volumetric flasks. Dilute to the mark in each of the six flasks using reagent grade water. Remember to use a plastic dropper to bring the bottom of the meniscus to the calibration mark. Fluorescence cuvettes have four transparent sides. If six fluorescence cuvettes are available, simply fill each plastic fluorescence cuvette with one of the prepared dye solutions. You should overlay each spectrum to allow easier comparison, by selecting "Store the previous run" after clicking the green Start button in Logger Pro. If only one cuvette is available, make sure to rinse the fluorescence cuvette several times with the new solution before taking the spectrum. Use only ChemWipes or soft tissue to dry the outside surfaces of the cuvette to prevent scratching. Don't place stirring rods or spatulas inside a cuvette, to prevent scratching. Make a Stern-Volmer plot to determine the quenching constant, K_Q.

5. *Clean-up*: Wash all your glassware with soap and water and leave all the items you used where you found them. Rinse your cuvettes with soap and water, taking care not to scratch the surfaces. You need not dry the cuvettes, just leave them to air dry (to avoid scratching).

Report:

You should use the **report form** to submit your laboratory report. The report should contain:

1. *Introduction*: Give a brief introduction. In the introduction state the purpose of the experiment and the principle means that you are going to use to achieve that purpose. The introduction should be just two sentences. You should not discuss the experimental procedure nor the calculations that will be necessary in the Introduction.

2. *Theory*: In a few sentences, report that the mechanism of quenching was determined by using the absorbance spectrum of the dye with and without quencher to determine the involvement of the ground state and a Stern-Volmer plot to determine the effectiveness of the quenching. Give the Stern-Volmer equation, Eq. 4, and identify each variable in words (I^o, I, K_Q, and $[I^-]$).

3. *Procedure*: Report the nature of your fluorescent dye sample: "a solution of fluorescein in pH 7 buffer prepared using the dye from a commercial highlighter." Give the manufacturer of the highlighter and model name. State that: "the absorbance spectrum of the dye without quencher and with 0.5 M I^- were determined. A Stern-Volmer plot was constructed with four KI concentrations from 0.1 to 0.4 M in 20% ethanol." Reference the instructions in this write-up. List any changes that were made to the written procedure in the lab write-up. State the manufacturer and model of the spectrophotometer. Report the excitation wave length for the fluorescence measurements.

4. *Results*: Provide a copy of your two absorption spectra with and without quencher. Provide the overlaid spectra of the fluorescence without quencher and with varying amounts of quencher. Present the results of the calculations for the Stern-Volmer experiment in a table: list the concentrations of I^-, the corresponding experimentally determined intensities, and I^o/I. Provide a copy of your Stern-Volmer plot. Give the slope and intercept of your plot and the corresponding quenching constant, K_Q. Make sure that each table and figure have captions and that each table or figure is referenced in the body of the text. The captions should summarize the content of the table or figure. The captions on the spectra can be hand written.

5. *Discussion*:
(a). Purpose accomplished: Restate the purpose as a completed goal (this sentence is just the introductory sentence for the first paragraph of the discussion).

(b). Final Results: State your conclusion on the interaction of the quencher with the ground state of the molecule: does the quencher interact with the ground state or the excited state of the dye molecule? Report the quenching constant, K_Q.

(c). Factors that influenced the precision and accuracy of the data:

i. Give a measurement that is source of random error. Which experimental measurement, the mass of KI, the volumes delivered by the auto-pipettor, the volume of the volumetric flasks, or the intensity of the fluorescence, gives the largest contribution to the error in the final result, K_Q. Does this predominant error have an effect on the accuracy or precision of the final result?

ii. Give a source of systematic error in the quenching constant: Do you have evidence for a systematic error? Give a suggestion for a source of systematic error (this is a difficult question, but give the question a try). Student mistakes are not systematic errors, they are just mistakes. Systematic errors are consistent for each trial.

(d). Answer the following question in your discussion:

1. Compare the absorbance spectrum of the dye with and without quencher, I^- ion. Are any significant changes in the absorption spectrum observed upon addition of I^- ion? Small changes in intensity might result from the relatively crude manner of solution preparation. What evidence do these observations provide for determining if the quenching mechanism is static or dynamic?
2. Compare the absorbance spectrum of the dye to the fluorescence spectrum. Which absorbance band corresponds to the excitation at 405 nm? Which transition, absorbance or fluorescence, is to smaller wavelength? Which transition is to higher frequency? In other words, which is bluer, which is redder? Which transition corresponds to greater energy changes?
3. Is the molecule excited by the incident light into the first or second excited state? Which excited state is responsible for fluorescence? Consider the excited state that is involved in the fluorescence transition. To what extent do the absorption spectrum of the excited state that is involved in the fluorescence transition and the fluorescence spectrum overlap? That is, are there wavelength regions that show both absorption and fluorescence emission?
4. Report the slope and intercept of the Stern-Volmer plot and the quenching constant K_Q. Is the quenching process adequately modeled by the Stern-Volmer mechanism? In other words, do you see evidence of systematic curvature or an intercept significantly different than 1.
5. Draw an energy level diagram similar to Figure 1. Label the vertical energy axis in both joules and kJ/mol. Using kJ/mol units will hopefully be meaningful in comparison with the energies involved in other chemical processes. Remember that the conversion is:

$$\Delta E = h\nu \, N_A \, (1 \text{ kJ}/1000 \text{ J}), \qquad \text{where } N_A \text{ is Avogadro's number} \qquad (5)$$

For the transitions, use the wavelength of maximum absorption or emission in the absorbance and fluorescence spectra. Include all observed excited states as determined from the absorption spectrum. In your energy level diagram include an arrow for the excitation using the 405 nm source, a dotted arrow for the internal conversion, and an arrow for the fluorescence transition back down to the ground state. The plot can be hand drawn.

Literature Cited

(1) Legenza, M.W. and Morzzacco, C.J., *J. Chem. Ed*., 54, 183, (1977).
(2) Bowen, E.J., *Trans. Faraday Soc*., 50, 97 (154).
(3) Stern, O., and Volmer, M., *Physik, Z.*, 20, 183 (1919).
(4) J. G. Calvert, J. N. Pitts, "Photochemistry", Wiley, New York, 1966, pp. 663-70.

SpectroVis Plus Spectrophotometers with Vernier Data Acquisition Software Instructions

Introduction:

The absorbance of a sample is given as $A = \log\left(\frac{I_0}{I}\right)$,

where I_o is the intensity without sample present and I is the intensity with the sample in the light path of the spectrophotometer. This calculation is done at each wavelength in the spectrum. The value of I_o depends on wavelength and must be determined by placing a reference or blank sample in the instrument. The terms reference and blank are used interchangeably. The reference is usually a cuvette filled with only the solvent or the buffer for the experiment. The process of acquiring the reference spectrum is called calibration in Logger Pro. Every time you switch solvents or buffers, a new reference spectrum must be taken, so the instrument must be recalibrated.

I. Getting Started and Calibration

1. Start the Logger *Pro* software.
2. The spectrophotometer should be automatically recognized by the software showing the colors of the visible spectrum in the data window with Wavelength as the horizontal axis. If the visible spectrum is not displayed, select Connect Interface →Spectrometer →Scan for Spectrometers from the Experiment menu.
3. Select Set Up Sensors →Spectrometer: from the Experiment menu. The Spectrometer dialog box will be displayed. Set Wavelength Smoothing to 0 and Samples to Average to 20. Using more Samples to Average gives more precise absorbances. Set the wavelength range to 480 – 700 nm. The Integration time will be set for you automatically when you do the calibration in the next step. Click on Close.

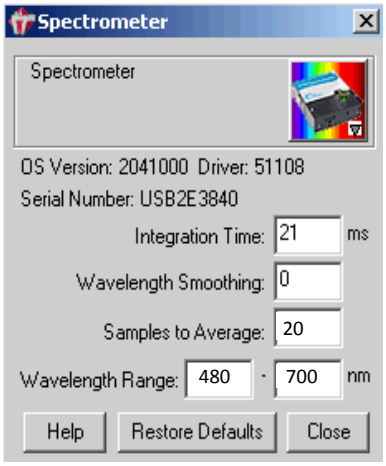


Figure 1. Settings for fast analysis with regular plastic cuvettes or work in the visible range.

4. To calibrate the Spectrometer, use a cuvette filled about ¾ full with water for your reference. Avoid touching the sides of the cuvette are in the light path. Choose Calibrate →Spectrometer from the Experiment menu. The calibration dialog box will display the message: "Waiting…60 seconds for lamp to warm up." (see Figure 2) The minimum warm up time is one minute. **NOTE: For best results, allow the spectrometer to warm up for at least five minutes.** Following the instructions in the dialog box to complete the calibration. Click Finish Calibration and then click OK.

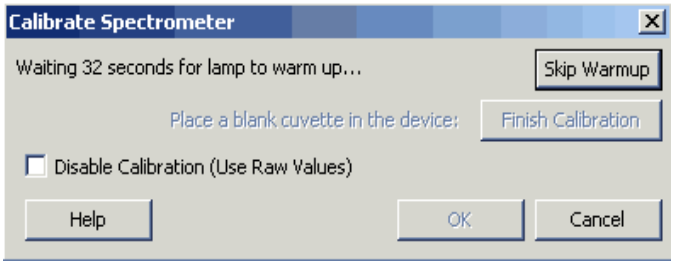


Figure 2. Calibration stores a reference spectrum.

After the first calibration, the lamp should be warmed up enough that you can skip the warm-up period on subsequent calibrations. The process of calibration sets the integration time for maximum sensitivity and also stores a reference spectrum.

II. Measuring Absorbance Spectra

1. Replace the reference cuvette with your sample. Click on 

and then 

.

2. You can read the absorbance using the Examine tool, by clicking on  . Then move the cursor along the spectrum. The wavelength and absorbance will be displayed in the new dialog box in the data window, Figure 3. Determine the wavelength of maximum absorbance. Use this same wavelength throughout your experiment.

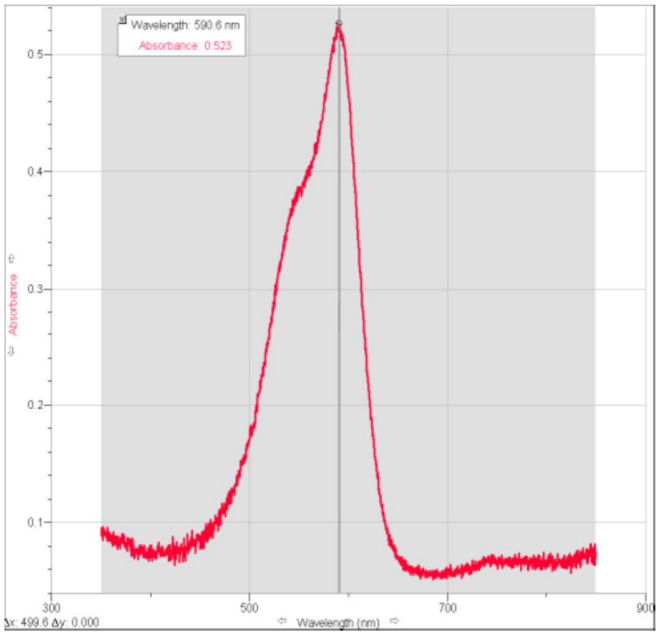


Figure 3. Spectrum of Crystal Violet with the Examine Tool.

Once you have determined the wavelength of maximum absorbance, you can use the Examine tool to read off the absorbance at that same wavelength and the absorbance at 700 nm for each sample. However, you might find it easier to read the absorbances from the spreadsheet output at the left of the screen. You can scroll through the spreadsheet data to determine the absorbance at the maximum wavelength and for the baseline at 700 nm.

3. ***Expanding the Axes:*** If you need to expand the x or y axis to see your spectrum better, use one of three methods:

Automatic scaling: Click on the Autoscale icon  .
Using the cursor: Position the cursor over the axis you want to expand. The cursor will change shape, Figure 4. Drag the mouse to change the scale expansion.

Figure 4. Move the cursor over the axis to change the axis scale.

Direct input: Click near the maximum or minimum of the axis you want to change. A dialog box will appear, Figure 5, and you can the type in the value that you want for the scale limit.

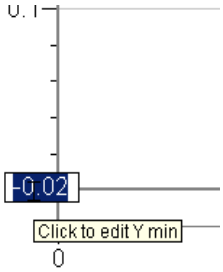


Figure 5. Click near the axis maximum or minimum to show the dialog box.

When working with multiple samples, you may choose to plot each spectrum separately or you can overlay successive spectra. Normally you plot each spectrum separately.

Plotting each spectrum separately:

4. Save the data file to the disk by pulling down the file menu and choosing Save As… Save your data files to the Documents directory.

5. Pull down the Data menu and choose Clear All Data.

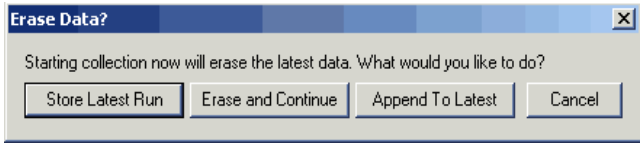
6. Replace the cuvette with another sample. You may need to calibrate again if there is a different reference solvent or buffer for the new sample. Return to step 1.

Overlayed Spectra

4. If you wish to overlay several spectra, choose Store Latest Run from the Experiment menu. This step allows you to overlay the next spectrum on top of the current spectra, with all showing. However, this step does not save the data file to the disk.

5. Replace the cuvette with another sample.

6. When you click on Collect this time, if you did not store the latest run to set-up for overlayed spectra in step 3, you will get a dialog box:



To overlay the next spectrum on top of the last spectra, click on Store Latest Run.

7. If you overlay your spectra, remember to save your combined spectra to disk. To save the data file to the disk pull down the file menu and choose Save As… Save your data files to the Documents directory.

III. Finishing up

1. Make sure to rinse your cuvettes three times with reagent grade water. Remember not to use paper towels to clean the plastic surfaces, only use ChemWipes. Don't stick anything sharp into the cuvettes, including a test tube brush.

2. Make sure the area around the spectrometer is clean and dry.